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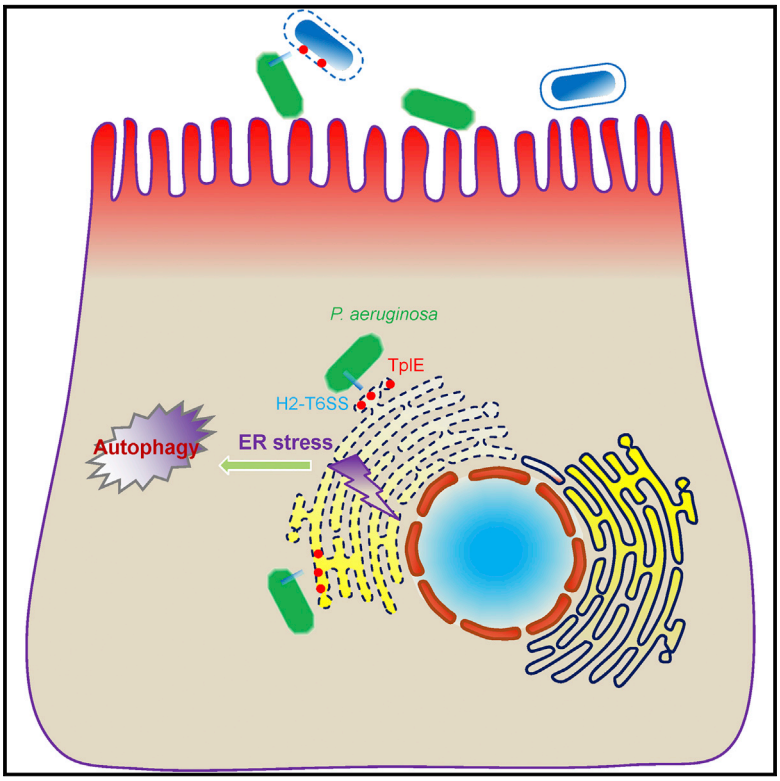
# The *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host Autophagy by Activating Endoplasmic Reticulum Stress

## Graphical Abstract




## Highlights

- TplE is an H2-T6SS dependent trans-kingdom effector of *P. aeruginosa*
- TplE is delivered to the bacterial periplasm for toxicity
- TplE contains a eukaryotic PGAP1-like domain for ER targeting
- Host cell ER stress and autophagy are induced by TplE translocation

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## In Brief

Jiang et al. report that the *P. aeruginosa* T6SS PGAP1-like phospholipase effector (TplE) targets the periplasm of competing bacteria to inhibit their growth. TplE can also target and disrupt the ER of eukaryotic cells, leading to ER stress and autophagic flux in the host cells.

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# The *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host Autophagy by Activating Endoplasmic Reticulum Stress

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## SUMMARY

***Pseudomonas aeruginosa*** **is an opportunistic pathogen that regularly causes nosocomial infections in hospitalized patients. The type VI secretion system (T6SS) is responsible for the secretion of numerous virulence effector proteins that can both interfere with competing microbes and manipulate host cells. Here, we report a detailed investigation of a *P. aeruginosa* H2-T6SS-dependent phospholipase effector, TplE, which acts as a trans-kingdom toxin. Delivery of TplE to the periplasmic space of rival bacteria leads to growth inhibition. Importantly, TplE, also contains a eukaryotic PGAP1-like domain, which targets the host ER apparatus, ultimately leading to disruption of the ER. TplE activity leads to the activation of the unfolded protein response (UPR) through the IRE1α-XBP1 pathway, enhancing autophagic flux. These findings indicate that this T6SS-delivered phospholipase effector is active against both prokaryotic and eukaryotic cellular targets, highlighting the T6SS as a versatile weapon in the *Pseudomonas* arsenal.**

## INTRODUCTION

Type VI secretion systems (T6SSs) are rapidly becoming some of most intensely studied secretion apparatus. They are widely distributed, including important pathogens such as *Vibrio*, *Pseudomonas*, and *Burkholderia* (Cianfanelli et al., 2016). T6SS have been shown to play critical roles in the interplay between adjacent bacteria, including inter- and intra-species killing, biofilm formation, and quorum sensing (Basler et al., 2013; Hood et al., 2010; Zoued et al., 2014). Moreover, T6SS have been proposed to be involved in bacterial pathogenesis by translocating effectors into eukaryotic cells, modulating host immunity and inflammation (Aubert et al., 2016; Jiang et al., 2014; Ma et al., 2009).

PGAP1 (post-glycosylphosphatidylinositol attachment to proteins 1) is a eukaryotic multi-spanning membrane serine-hydrolase normally localized on the ER, which possesses a conserved lipase motif (GxSxG). This protein mediates inositol deacylation, which is important for efficient ER-Golgi trafficking. PGAP1 and its homologs are encoded by a large number of eukaryotic species, ranging from mammalian cells to yeast. Mutation of the catalytic serine residue of PGAP1 protein to alanine causes a complete loss of function (Tanaka et al., 2004).

The ER is an important eukaryotic organelle involved in multiple physiological functions. Perturbation of ER homeostasis leads to stress and subsequently activation of a series of complementary mechanisms called the unfolded protein response (UPR). The UPR comprises three signaling pathways that are initiated by the stress sensors protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (Hetz, 2012). Evident traits for UPR induction comprise the upregulation of ER molecular chaperone GRP78 (also referred to as Bip) expression, induction of the transcription factor CHOP, and the splicing of XBP1 mRNA (Li et al., 2008). If homeostasis cannot be restored, then autophagy is initiated and cell death ultimately occurs (Hetz, 2012).

Autophagy is a highly conserved cellular activity that engulfs cytosolic material into double-membrane vesicles that are eventually targeted toward lysosome fusion for degradation (Mizushima and Komatsu, 2011). Autophagy vesicles are formed from the ER membrane and, upon subsequent maturation, play a central role in homeostasis of cell metabolism by removing damaged organelles and misfolded proteins (Lamb et al., 2013). Autophagy has been shown to play a role in the interaction with diverse intracellular pathogens (Huang and Brumell, 2014). Although *Pseudomonas aeruginosa* has been previously reported to induce autophagy in various cell lines (Junkins et al., 2013; Yuan et al., 2012), the mechanism by which it does this remains unclear.

In this study, we have identified a *P. aeruginosa* trans-kingdom T6SS effector, PA1510 (designated TplE for type 6 PGAP1-like effector). TplE is a Tle4 phospholipase family protein (Russell et al., 2013) that facilitates inter-bacterial killing in addition to inducing a PGAP1-domain-dependent autophagy activation through disruption of ER homeostasis.

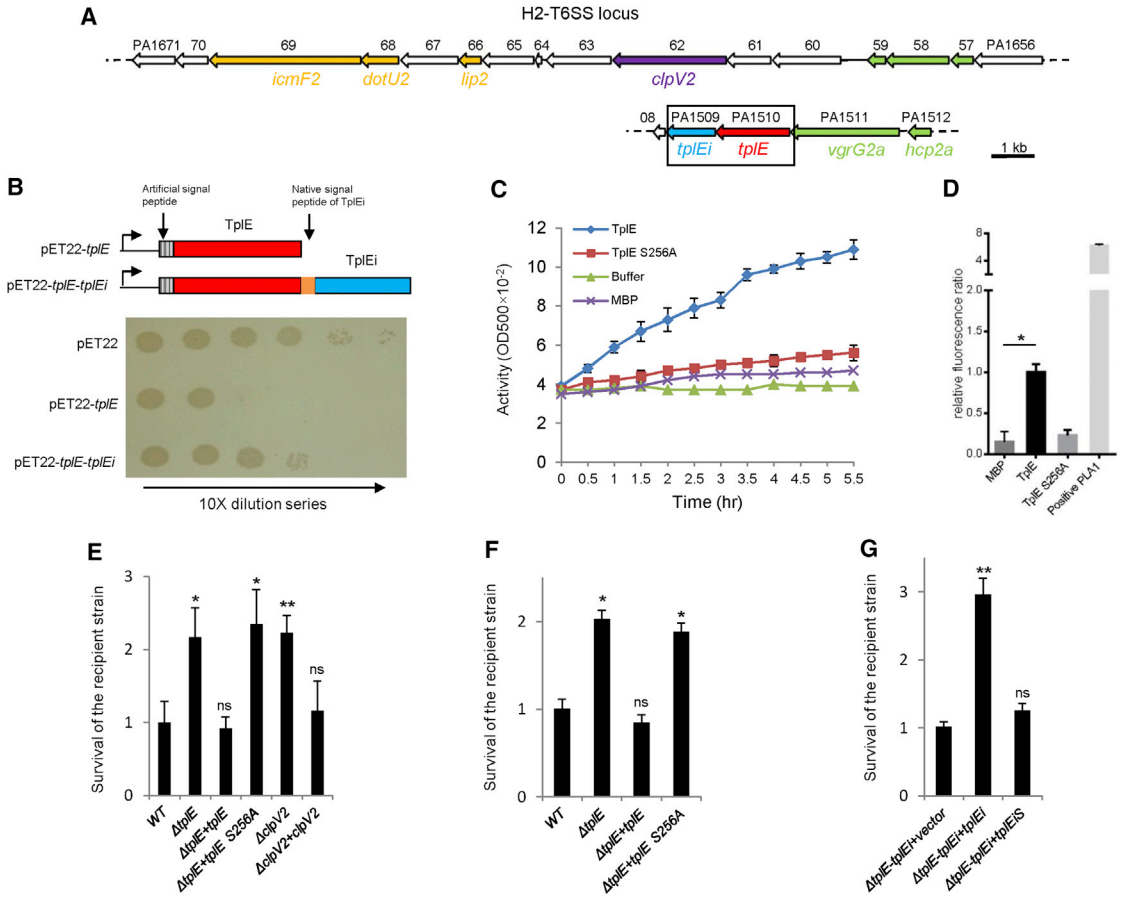


**Figure 1. Identification of an H2-T6SS-Dependent Lipase Effector in *P. aeruginosa***

(A) The H2-T6SS locus of *P. aeruginosa* PAO1. Genes *tplE* and *tplEi* (red and blue fill, respectively) are encoded downstream of *vgrG2a*. Membrane-associated proteins are shown in yellow, tailed bacteriophage proteins in green, and ATPase in purple.

(B) Heterologous periplasmic production of TplE using pET22b inhibits growth of *E. coli*. Co-production of the TplEi immunity protein partially protects against this inhibitory effect.

(C) The lipase activity of purified MBP, MBP-TplE, and MBP-TplE S256A was determined by digestion of Tween-20.

(D) Phospholipase A1 activity of MBP, MBP-TplE, and MBP-TplE S256A was measured using PED-A1 as a substrate (Invitrogen).

(E–G) Intra- and inter-species growth competitions. (E) Intra-species competition using the PAO1 *ΔtplE-tplEi::Gm* strain as the recipient and attacker strains as indicated. (F) Inter-species competition using *P. putida* KT2440 as the recipient and attacker strains as indicated. (G) Complementation of TplEi or TplEiS in intra-species competition experiments. All survival data were normalized to the WT or vector, respectively. ANOVA analysis was applied with WT or vector as the comparator. *$p < 0.01$; **$p < 0.05$; ns, not significant.

See also Figure S1.

## RESULTS

### TplE Is an H2-T6SS-Dependent Antibacterial Lipolytic Toxin

*P. aeruginosa* PAO1 encodes three T6SS loci, designated:H1, H2 and H3-T6SS. VgrG2a (PA1511) and Hcp2a (PA1512) are known to be key components of the H2-T6SS apparatus, although they are not tightly linked to the other H2-T6SS genes PA1656–PA1671 (Barret et al., 2011; Mougous et al., 2006). VgrG2a was demonstrated to be required for the H2-T6SS-mediated translocation of VgrG2b, a T6SS virulence effector, into host cells (Sana et al., 2015). Previously a putative T6SS lipase effector (PA1510) with a tightly linked potential immunity protein gene (PA1509) were identified downstream of VgrG2a, now designated TplE and TplEi, respectively (Figure 1A). TplE belongs to the Tle4 lipase family (Russell et al., 2013), although until now, its biological role has remained unresolved.

As TplE encodes a conserved catalytic triad (S-H-D) and a TxSxG motif (Lu et al., 2014), we hypothesized that it would exhibit a lipase-dependent antibacterial activity. *Escherichia coli* toxicity assays were conducted to test this. While heterologous cytoplasmic production of TplE in *E. coli* did not lead to growth inhibition (Figures S1A and S1B), periplasm targeting of TplE resulted in a significant inhibition of growth (Figures 1B and S1B). Importantly when we co-synthesized the putative immunity protein TplEi and targeted it to the periplasm, we observed a repression of the TplE-dependent growth inhibition by up to two orders of magnitude (Figures 1B, S1C, and S1D).

In order to test the biochemical activity of TplE, we constructed an allele with a site-directed mutation in the hypothetical active site (S256A). We heterologously expressed the wild-type (WT) and catalytic mutant alleles as N-terminal maltose-binding protein (MBP) fusions and as C-terminal hexahistidine tag fusions. The purified proteins were tested for their ability to hydrolyze Tween-20, which is a classical substrate for lipase activity. While TplE demonstrated clear lipase activity, the activity of the TplE S256A mutant was significantly decreased (Figure 1C), confirming the functionality of the predicted lipase active site of TplE. We found that TplE also exhibited lipolytic activity when we used long-chain triglycerides such as olive oil or triolein as substrates (Figure S1E). Next, we tested whether TplE possesses phospholipase activity. Using substrates specific for phospholipase A1 (PLA1) or A2 (PLA2), we observed that TplE acts specifically as a PLA1 but not as a PLA2; this PLA1 activity was abolished by S256A mutation (Figures 1D and S1F).

The contribution of TplE was investigated in intra- and inter-species competition studies. Intra-species competition assays between the PAO1 *ΔtplE-tplEi::Gm* "recipient" strain and various "attacker" derivatives confirmed that the loss of *tplE* gave rise to a growth advantage of the recipient strain. This advantage was lost by trans-complementation of the PAO1 *ΔtplE* strain with intact *tplE*, but not with *tplE* S256A (Figure 1E). Furthermore, specific inactivation of H2-T6SS (*ΔclpV2*) in the attacker strain diminished the TplE-dependent toxicity, indicating that this system is responsible for TplE delivery (Figure 1E).

We saw analogous results in inter-species competition experiments between *P. aeruginosa* and *Pseudomonas putida*. Deletion of *tplE* reduced the ability of *P. aeruginosa* attacker to out-compete *P. putida* recipient. Trans-complemented with *tplE*, but not with *tplE* S256A, the competitive advantage was restored (Figure 1F).

Finally, trans-complementation with the immunity protein gene *tplEi* rescued the fitness defect of the PAO1 *ΔtplE-tplEi* strain when competed against the WT strain. As expected, this effect was lost when we complemented with an allele of *tplEi* without the N-terminal signal sequence (*tplEiS*). TplEiS could not reach the periplasm, which is the site at which it can provide protection against the phospholipase activity of incoming TplE, being delivered by the attacker strain (Figures 1G and S1G). Moreover, co-immunoprecipitation experiments confirmed that TplE and TplEi do physically interact (Figure S1H; see also Lu et al., 2014). Taken together, these data confirmed the role of TplE as an H2-T6SS-dependent antibacterial effector that is functional based on its phospholipase A1 activity and the role of TplEi as a cognate periplasmic immunity protein.

## TplE Encodes a Eukaryotic PGAP1-like Domain and Can Be Translocated into Host Cells

Bioinformatic analysis predicted that TplE encodes a PGAP1-like domain. Multiple sequence alignments with prokaryotic Tle4 family members and eukaryotic PGAP1 domain (CDD: pfam07819) indicated that these proteins possess a PGAP1-domain core region (Figure 2A). Phylogenetic analysis of 79 PGAP1-like proteins from a wide range of eukaryotic and prokaryotic species suggest evolutionary divergence based primarily around differences in their PGAP1 domains (Figures 2B and S2).

To test whether *P. aeruginosa* could translocate TplE into HeLa cells, we infected HeLa cells with different *P. aeruginosa* strains that were producing the TplE::Bla fusion protein. We then applied CCF2-AM, which is a green fluorescent substrate that can freely enter host cells. When processed by β-lactamase, this substrate switches to blue fluorescence, thus acting as a marker for the TplE::Bla protein. In these experiments, HeLa cells exhibited a higher blue fluorescence when exposed to the *P. aeruginosa* strain producing the TplE::Bla protein, confirming TplE translocation into epithelial cells (Figures 2C and 2D). TplE translocation was abolished when the *P. aeruginosa ΔclpV2* strain was used and could be restored by trans-complementation of a functional *clpV2* gene (Figures 2C and 2D). These results confirm that TplE can be translocated into epithelial cells in an H2-T6SS-dependent manner.

## TplE Co-localizes with the ER and Leads to ER Disruption

Given that eukaryotic PGAP1 proteins are located at the ER membrane, we asked whether TplE would also localize to the ER. However, due to differences in the codon usage between prokaryotic and eukaryotic cells, the expression level of the *tplE* gene was low in mammalian cells when using the native *tplE* sequence (Figures S3B and S3C). Therefore we re-synthesized *tplE* as a eukaryotic codon-optimized allele (Figure S3A), which then gave sufficient expression levels for localization studies (Figures S3B and S3C). The optimized *tplE* gene with a C-terminal mRFP gene fusion was constructed and transfected into HeLa cells, the cells were then stained with ER-Tracker green dye to detect co-localization of the ER with the red-fluorescent TplE fusion. The fusion protein and ER were seen to co-localize around the cell nucleus (Figure 3A, top). A TplE S256A-mRFP fusion protein was also observed co-localization with the ER, suggesting enzymic activity was not involved in localization to the ER (Figure 3A, middle).

In order to determine whether TplE co-localizes with the host cell ER during *P. aeruginosa* infection, we infected HeLa cells with various *P. aeruginosa* strains producing FLAG-tagged TplE. Since TplE S256A mutant also targets the host ER (Figure 3A) but induces less cytotoxicity than the WT TplE, we used strains producing this mutated protein to minimize toxic effects on ER. Immunostaining revealed that translocated TplE S256A-FLAG localized to ER in an H2-T6SS-dependent manner (Figures 3B and 3C). Similar results were obtained when using an alternative ER staining method (Figure S4A). These data suggest that the H2-T6SS effector TplE is targeted to host ER during bacterial infection.

Next, we examined the effect that TplE has upon the ER. HeLa cells producing CALR-GFP for ER labeling were used in this assay. Transient expression of *tplE* gene resulted in a contraction of the ER surrounding the nuclear periphery when compared to the vector-only control, which showed the regular thick tubular reticular ER structure (Figure 3D). Approximately 70% of cells producing TplE displayed ER disruption compared to 20% of control cells (Figure 3E). Furthermore, transient expression of

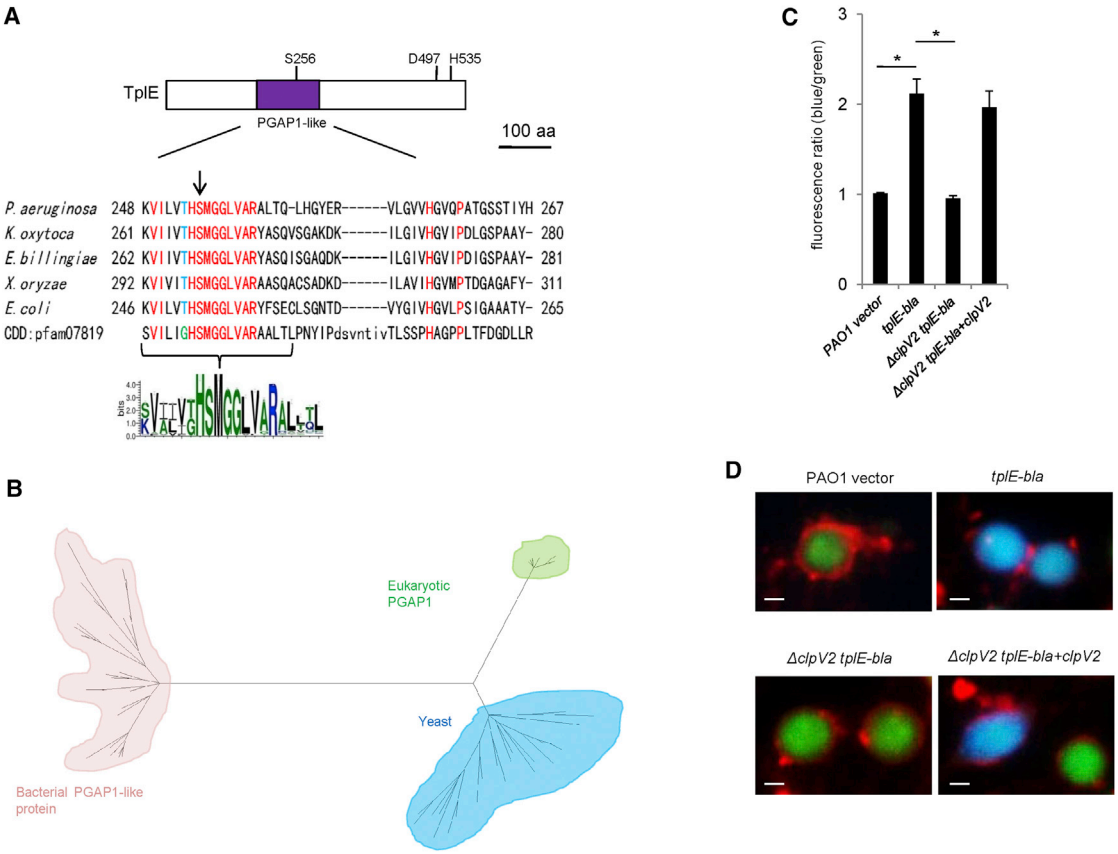


**Figure 2. TplE Encodes a Eukaryotic PGAP1-like Domain and Is Active against Mammalian Cells**

(A) Sequence alignments of prokaryotic Tle4 members encoding PGAP1-like domain. The consensus eukaryotic PGAP1 domain (pfam07819) is shown below and the conserved catalytic residue Ser256 is indicated by the arrow.

(B) A phylogenetic tree of PGAP1 proteins from different taxa. The maximum-likelihood method was used to generate phylogenetic tree from 79 PGAP1 sequences.

(C and D) Translocation of TplE::Bla into host cells. (C) Cultured HeLa cells were co-incubated with various *P. aeruginosa* strains (x axis), producing the TplE::Bla fusion. The ratio of blue fluorescence (450 nm) to green fluorescence (520 nm) is shown. (D) Fluorescent microscopic examination of these same HeLa/*Pseudomonas* strain combinations. The positions of the cell membrane were indicated by plasma membrane dye (Invitrogen). Scale bars, 10 μm. *$p < 0.01$. See also Figure S2.

the *tplE* S256A gave a similar pattern of ER distribution to that of the control cells, suggesting disruption of ER structure requires the catalytic activity of TplE (Figures 3D and 3E).

Finally, we tested whether this ER-disruption phenotype could be induced through the more physiologically relevant route of TplE translocation directly from *P. aeruginosa*. The ER of HeLa cells infected with the *P. aeruginosa* WT was seen to be disrupted. However, this effect was reduced significantly when we used *ΔtplE* strain. Trans-complementation of the mutant with a functional *tplE* allele, but not with *tplE* S256A, restored the ER-disruption phenotype (Figures 3F and 3G). The control experiments with *P. aeruginosa* T6SS (*ΔclpV2*) or T3SS (*ΔpcrV*) knockout strains showed that this phenotype is dependent on the T6SS effector TplE, but not on the T3SS effectors (Figures 3F and 3G). LDH release assays under the same conditions confirmed that TplE did not promote cytotoxicity (Figure S4B). These results indicate that TplE targets the ER compartment, leading to ER disruption mediated by phospholipase activity.

## TplE Induces the Unfolded Protein Response and Autophagy

It is known that ER stress induces the binding of chaperones Bip/Grp78 to misfolded proteins and thus initiates the UPR (Hetz, 2012). We therefore hypothesized that TplE-dependent ER disruption would also result in UPR activation. We used transient expression of *tplE* gene in HEK293T cells to test this. As a positive control for ER stress and UPR activation, we treated non-transfected cells with thapsigargin, which is an inhibitor that blocks $Ca^{2+}$ uptake to the ER (Li et al., 2008). As biomarkers for ER stress, we first monitored the levels of Bip and CHOP proteins. Our results showed that transient expression of *tplE* gene, but not *tplE* S256A, led to an upregulation of Bip and CHOP (Figure 4A). Next, we examined activity of the ER protein IRE1α. In this case, activated IRE1α catalyzes the splicing of a 26-nt intron from XBP1 mRNA (XBP1u). This processed mRNA form allows the synthesis of the active XBP1s transcriptional factor, which is responsible for terminating the ER stress response (Yoshida

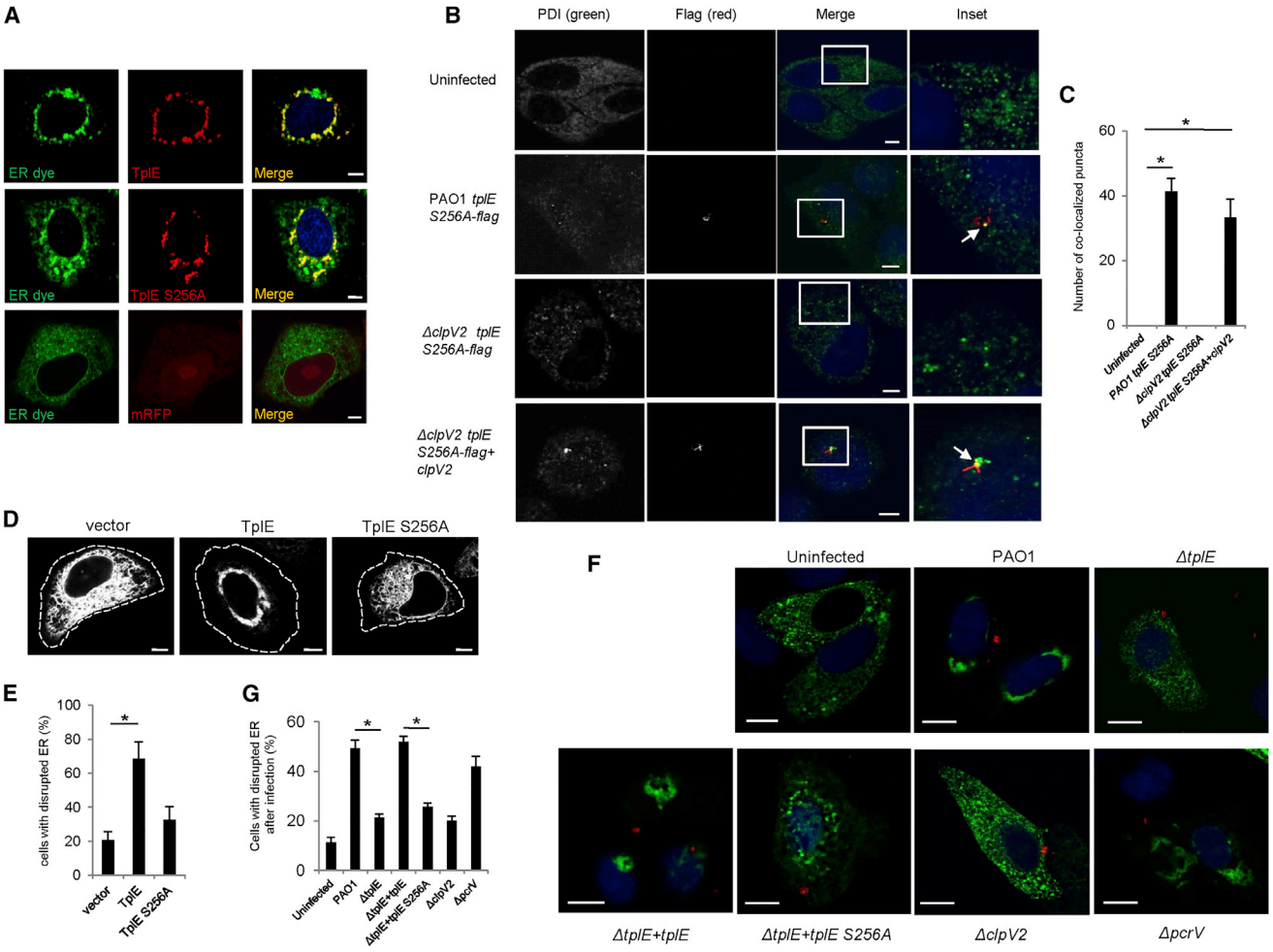


**Figure 3. TplE Co-localizes with the ER and Causes ER Disruption**

(A) TplE localizes to the ER. HeLa cells were transfected with plasmids containing a TplE::mRFP fusion, a TplE-S256A::mRFP fusion, or the mRFP gene alone. Scale bars, 5 μm.

(B and C) TplE targets the host ER upon *P. aeruginosa* infection. (B) HeLa cells were infected or not (uninfected) with the indicated PAO1 strains producing TplE S256A-FLAG. TplE was detected by anti-FLAG (red) and ER by anti-PDI (protein disulfide isomerase, an ER-specific marker protein, green). Scale bars, 5 μm. (C) Shown is the number of co-localized puncta in 300 cells from three independent experiments (mean ± SEM).

(D and E) TplE induces ER disruption in HeLa cells. (D) The effect of transient expression of *tplE* and *tplE* S256A on ER integrity was assessed in CALR-GFP-producing cells. Borders of representative cells are highlighted. Scale bars, 5 μm. (E) Quantification of the numbers of cells exhibiting contracted ER. Experiments were repeated in triplicate. Error bars represent mean ± SEM (n = 100).

(F and G) TplE-dependent ER disruption in HeLa cells during bacterial infection. (F) CALR-GFP-producing HeLa cells were infected with the indicated *P. aeruginosa* strains, and the architecture of the ER was examined. *Pseudomonas* bacteria are labeled red with anti-*Pseudomonas* antibody. (G) Quantification of the percentage of cells with disrupted ER after infections. Error bars represent mean ± SEM (n = 50). *p < 0.01.

See also Figures S3 and S4.

et al., 2001). We determined the ratio of XBP1u and XBP1s mRNA transcripts from treated cells. Results show that cells producing active TplE, but not the S256A mutant, generated the processed XBP1s mRNA significantly (Figures 4B and 4C). These results show that TplE induces the ER stress response by upregulating the expression levels of Bip and CHOP and inducing the splicing of XBP1 mRNA.

It was previously reported that ER stress can act as a potent trigger for autophagy (Yorimitsu et al., 2006). We investigated the effect of transient expression of *tplE* gene on the conversion of LC3-I to LC3-II in HEK293T cells; this conversion is a hallmark of autophagy induction. We confirmed that WT TplE, but not the S256A mutant, was able to induce significant levels of LC3-II production (Figure 4D). The degradation of p62, an LC3-binding protein that accumulates in autophagy-deficient cells, was enhanced by TplE, but not by its mutant (Figure 4D). These results suggest that the enzymic activity of TplE is required for autophagy induction. To test the ability of TplE to induce autophagy under more physiologically relevant conditions, we performed infection experiments with various *P. aeruginosa* strains. In these experiments, rapamycin-treated cells acted as a positive control. Consistent with the transient expression studies,

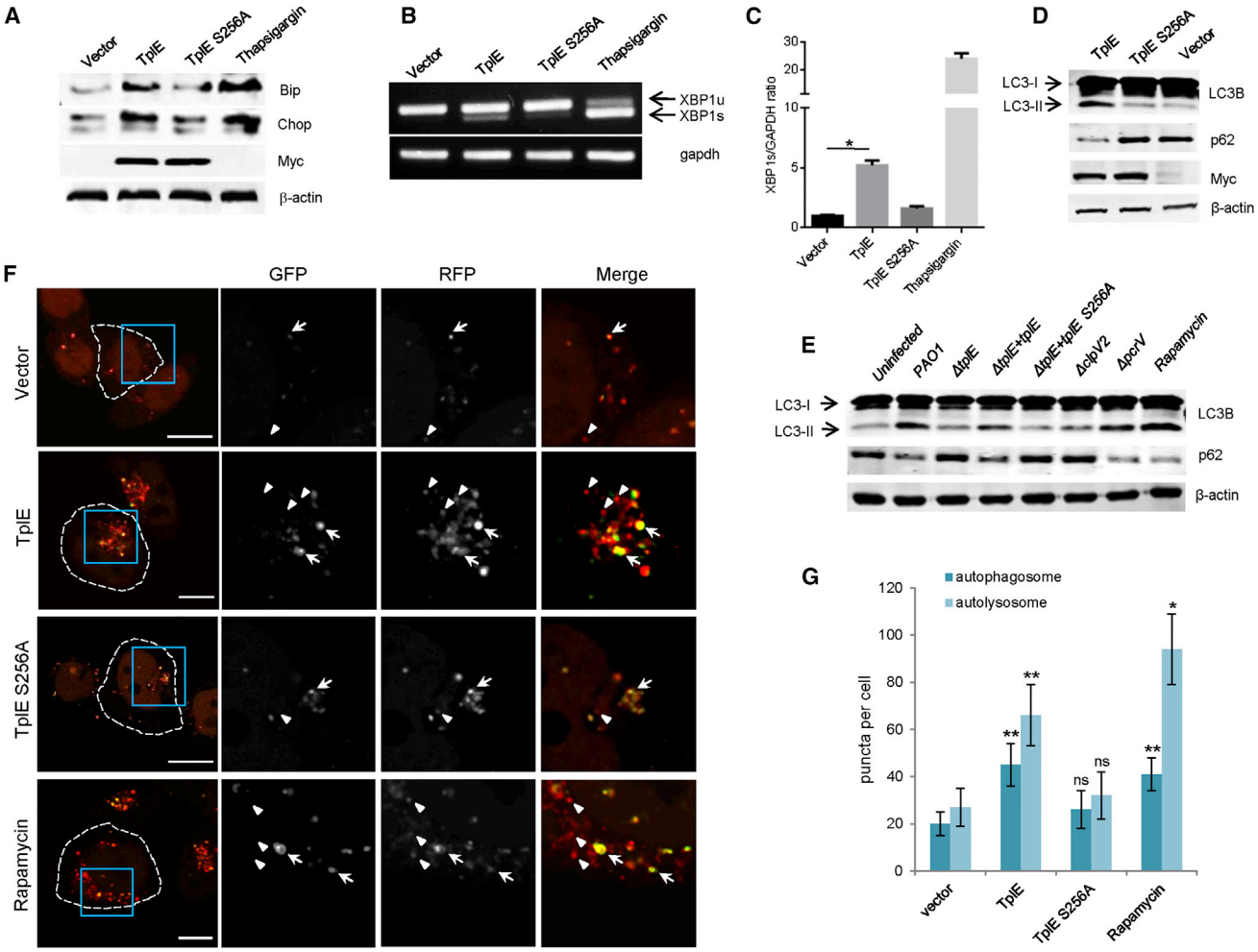


**Figure 4. TplE Induces the Unfolded Protein Response and Autophagy**

(A) HEK293T cells were transfected with indicated expression plasmids. Expression levels of the Bip and CHOP were determined. As a positive control for UPR induction, cells were treated with 300 nM thapsigargin for 4 hr.

(B and C) TplE induces splicing of XBP1 mRNA transcripts. (B) TplE, but not its catalytic mutant, TplE S256A, induces splicing of XBP1 transcripts in HEK293T cells. The XBP1 transcript size was determined using RT-PCR. XBP1u, unspliced; XBP1s, spliced; *gapdh*, loading control. (C) The intensity of the XBP1s was quantified. Data are from three independent experiments. Error bars represent mean ± SEM.

(D and E) TplE induces autophagy upon transfection or infection. (D) WT *tplE* and *tplE* S256A mutant were transiently expressed in HEK293T cells and cell lysates were immunoblotted for LC3-I, LC3-II and p62. (E) Immunoblotting of lysates from *P. aeruginosa*-infected HeLa cells with LC3B and p62 antibodies. 1 μM rapamycin treatment for 1 hr was included as a positive control.

(F and G) Imaging and quantification of TplE-induced autophagy. HeLa cells stably expressing mRFP-EGFP-LC3B were transfected with indicated plasmids. (F) Representative confocal images illustrating the presence of autophagosomes ($RFP^+GFP^+$ yellow dots, arrows) and autolysosomes ($RFP^+GFP^-$ red dots, triangles). (G) The numbers of yellow and red puncta per cell were quantified using ImageJ. Results were calculated from triplicate experiments. Error bars represent mean ± SEM. ANOVA analysis was applied with vector as the comparator. *$p < 0.01$; **$p < 0.05$; ns, not significant.

β-actin served as a loading control in (A), (D), and (E). Myc blot in (A) and (D) was included to ensure equal amount of TplE and TplE mutant production. See also Figure S3.

we observed that cells infected with PAO1 induced processing of LC3-I to LC3-II. The levels of LC3-II produced were higher in the WT strain than in the *ΔtplE* derivative. This level of LC3-II production was restored when the mutant was trans-complemented with the functional *tplE* allele, but not with the S256A mutant (Figure 4E). The degradation of p62 paralleled the changes in LC3-II observed with the WT and *ΔtplE* strains. Moreover, the efficient autophagy induction by *ΔpcrV*, but not by *ΔclpV2*, suggested that T3SS might not be required for increased autophagy following *P. aeruginosa* infection (Figure 4E). These results established that the autophagic flux is induced by TplE delivery.

Finally, to determine the impact of TplE on autophagy at cellular level, a tandem expression vector producing mRFP-EGFP-LC3B fusion protein was utilized. Cells stably expressing this tandem construct were transfected with plasmids producing TplE or the S256A mutant. Both cells were shown to produce same amount of TplE protein (Figure S3D). We then monitored mRFP-EGFP-LC3B expression by examining the fluorescence

colors of any cellular puncta formed. In this case, red puncta represent autolysosome formation, as the red mRFP remains visible while the green EGFP is quenched due to acidic conditions within the lysosome. Alternatively, yellow puncta represent autophagosome formation, as both the red mRFP and green EGFP can be detected simultaneously. Results showed that in cells transfected with *tplE*, the number of autophagosome and autolysosome puncta increased compared to either the vector control or in *tplE* S256A-tranfected cells (Figures 4F and 4G). Cells treated with rapamycin, an inducer of the autophagy flux, were provided a positive control (Figures 4F and 4G). These observations indicate that the enzymic activity of TplE can lead to induction of autophagy flux in human epithelial cells.

## DISCUSSION

Increasing numbers of T6SS-dependent effector proteins are being characterized (Alcoforado Diniz et al., 2015). More recently, many T6SS lipase family effectors have been studied for their eukaryotic cell targeting functions. The *P. aeruginosa* phospholipase D effectors PldA and PldB were proven to be instrumental for bacterial invasion of eukaryotic host cells (Jiang et al., 2014). A VgrG2b protein was recently demonstrated to be also involved in *P. aeruginosa* internalization of epithelial cells; this was the initial described phenotype for H2-T6SS of PAO1 (Sana et al., 2015; Sana et al., 2012).

In this study, we characterized the activities of an H2-T6SS-dependent phospholipase effector, TplE, which encodes a eukaryotic PGAP1-like domain. In addition to exerting a toxic effect in the periplasmic space of adjacent bacteria, this trans-kingdom effector can also be injected into eukaryotic host cells. In mammalian cells, TplE becomes localized to the ER, whereupon it causes stress and the UPR and ultimately triggers an autophagy response.

The apparent contributions of *P. aeruginosa* H2-T6SS in such diverse processes as bacterial internalization and autophagy induction are intriguing. Internalization is considered to be the initial step and the most frequently used tactic employed by the invasive bacteria to evade innate immunity of eukaryotic hosts. *P. aeruginosa* uses the H2-T6SS effectors PldA and VgrG2b to promote actin- or microtubule-dependent entry into epithelial cells (Jiang et al., 2014; Sana et al., 2015). Once pathogens are engulfed, they come into contact with different cellular organelles (Escoll et al., 2016). In response to pathogen invasion, host cells mount innate immune responses such as autophagy to combat these bacteria. Multiple lines of evidence have proved the existence of an active interplay between bacterial effectors and host autophagy (Huang and Brumell, 2014). Considering that T6SS acts in a contact-dependent manner (Russell et al., 2011), it is possible that intracellular *P. aeruginosa* comes into contact with the ER apparatus, activates the H2-T6SS machinery, and targets its effector TplE into the ER, causing subsequent cytotoxicity. However, it still remains unclear what signals are needed for the discriminative attack of T6SS toward eukaryotic cells. In addition, although previous studies demonstrated that *P. aeruginosa* infection induces autophagy in mammalian cells and that this response plays a vital role in clearing intracellular bacteria (Junkins et al., 2013; Yuan et al., 2012), it remains to be determined whether the host cell response to TplE constitutes an attempt to clear bacterial cells or a mechanism to protect against cell death.

A recent study suggested that *P. aeruginosa* infection leads to activation of UPR pathways (van 't Wout et al., 2015). The work we present here confirms this observation and identifies the H2-T6SS-dependent TplE effector as the direct mediator of this effect, eliciting an increase in the expression of the ER chaperones Bip and CHOP. In addition, TplE was shown induce splicing of XBP1 mRNA in human epithelial cells, suggesting that the TplE-induced UPR is dependent on the IRE1$\alpha$-XBP1 signaling pathway. Consistent with this is the previously published observation that the XBP1 branch of the UPR possesses a conserved role for host cell protection from *P. aeruginosa* infection (Richardson et al., 2010).

Although the GPI inositol-deacylase activity of TplE was not validated in this study, it is still possible that TplE shares similar functions as mammalian PGAP1 proteins for ER targeting. Unlike the host PGAP1 protein, it is likely that TplE's phospholipase activity would not be regulated by the host cell. Indeed, mutation of the key active site residue, TplE S256A, confirmed that phospholipase activity is critical to TplE's influence on both prokaryotic competition and host cell phenotypes.

Our observations illustrate how a single T6SS effector protein can contribute to the complex interplay among a bacterial pathogen, competing microbes, and the host cell during an infection. It will facilitate further exploration of how *P. aeruginosa* utilize its T6SSs and arsenal of effectors for manipulating the host cell and potentially lead to strategies for designing approaches to combat bacterial infections.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains, Plasmids, Cell Lines, and Growth Conditions

All bacterial strains and plasmids used in this study were listed in Table S1. Details of strain, plasmid constructions, growth condition of bacteria and cell lines are described in the Supplemental Experimental Procedures and Table S1.

### Growth Competition Assays

Intra- and inter-species growth competition assays were performed as described previously (Jiang et al., 2014). For *P. aeruginosa-P. aeruginosa* competition, the initial ratio was 5:1 (attacker:recipient) and incubation for 24 hr at 37°C. For *P. aeruginosa-P. putida* competition, the initial ratio was 1:1 (attacker:recipient) and incubation for 24 hr at 30°C. Detailed information can be found in the Supplemental Experimental Procedures.

### Transient Transfection and Confocal Microscopy

HeLa cells were cultured 24 hr in DMEM with 10% fetal bovine serum (FBS), and transient transfection was performed using Lipofectamine 2000 in Opti-MEM medium following the manufacturer's instructions (Invitrogen). All confocal images were taken on a Leica TCS SP5 microscopy. Images were processed using ImageJ software. Full details are in the Supplemental Experimental Procedures.

### Bacterial Infection and Cytotoxicity Assays

HeLa cells were cultured in DMEM with 10% FBS in 12-well plates. When grown to 70% confluence at 37°C, cells were washed twice with PBS and infected with *P. aeruginosa* strains from stationary phase at an MOI of 100 for 4 hr. Subsequently, the cell cultures were washed twice and then applied for subsequent translocation assays, confocal observations, or western blot analysis. For cytotoxicity assays, cells in 96-well plate were infected for 4 hr, and

any LDH released was assessed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega).

### Statistical Analysis

One-way ANOVA or two-tailed Student's t test was used to confirm statistical significance at 95% confidence. If one-way ANOVA was performed, differences were analyzed by a subsequent Bonferroni's post hoc test. A p value $< 0.05$ was considered to be significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.012.

## AUTHOR CONTRIBUTIONS

F.J., G.Y., and Q.J. conceived the project. F.J., X.W., and G.Y. performed the experiments. F.J., X.W., B.W., L.C., Z.Z., N.R.W., and G.Y. analyzed the data. F.J., N.R.W., and G.Y. wrote the paper.

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